

NOVEL MEGAKARYOCYTIC PROTEIN TYROSINE KINASES

Sub a13 This application is a continuation application of United States Application Serial Number 08/232,545, filed April 22, 1994, which is incorporated herein in its entirety.

1. Introduction

The present invention relates to novel cytoplasmic tyrosine kinases isolated from megakaryocytes (megakaryocyte kinases or MKKs) which are involved in cellular signal transduction pathways and to the use of these novel proteins in the diagnosis and treatment of disease.

The present invention further relates to specific megakaryocyte kinases, designated MKK1, MKK2 and MKK3, and their use as diagnostic and therapeutic agents.

2. Background

Cellular signal transduction is a fundamental mechanism whereby external stimuli that regulate diverse cellular processes are relayed to the interior of cells. These processes include, but are not limited to, cell proliferation, differentiation and survival. Many tyrosine kinases are expressed in postmitotic, fully differentiated cells, particularly in the case of hematopoietic cells, and it seems likely that these proteins are involved in specialized cellular functions that are specific for the cell types in which they are expressed. (Eiseman, E. and J.B. Bolen, Cancer Cells 2(10):303-310, 1990). A central feature of signal transduction is the reversible phosphorylation of certain proteins. (for reviews, see Posada, J. and Cooper, J.A., 1992, Mol. Biol. Cell 3:583-392; Hardie, D.G., 1990, Symp. Soc. Exp. Biol. 44:241-255). The phosphorylation state of

a protein is modified through the reciprocal actions of tyrosine kinases (TKs), which function to phosphorylate proteins, and tyrosine phosphatases (TPs), which function to dephosphorylate proteins.

- 5 Normal cellular function requires a delicate balance between the activities of these two types of enzyme.

Phosphorylation of cell surface tyrosine kinases, stimulates a physical association of the activated receptor with intracellular target molecules. Some of
10 the target molecules are in turn phosphorylated. Other target molecules are not phosphorylated, but assist in signal transmission by acting as adapter molecules for secondary signal transducer proteins.

- The secondary signal transducer molecules
15 generated by activated receptors result in a signal cascade that regulates cell functions such as cell division or differentiation. Reviews describing intracellular signal transduction include Aaronson, S.A., Science 254:1146-1153, 1991; Schlessinger, J.
20 Trends Biochem. Sci. 13:443-447, 1988; and Ullrich, A., and Schlessinger, J. Cell 61:203-212, 1990.

- Receptor tyrosine kinases are composed of at least three domains: an extracellular ligand binding domain, a transmembrane domain and a cytoplasmic
25 catalytic domain that can phosphorylate tyrosine residues. The intracellular, cytoplasmic, non-receptor protein tyrosine kinases may be broadly defined as those protein tyrosine kinases which do not contain a hydrophobic, transmembrane domain. Bolen
30 (Oncogene, vol. 8, pgs. 2025-2031 (1993)) reports that 24 individual protein tyrosine kinases comprising eight different families of non-receptor protein tyrosine kinases have been identified: Abl/Arg; Jak1/Jak2/Tyk2; Fak; Fes/Fps; Syk/Zap; Tsk/Tec/Atk;
35 Csk; and the Src group, which includes the family members Src, Yes, Fyn, Lyn, Lck, Blk, Hck, Fgr and

Yrk. All of the non-receptor protein tyrosine kinases are thought to be involved in signaling pathways that modulate growth and differentiation. Bolen, *supra*, suggests that half of the nonreceptor
5 protein tyrosine kinases have demonstrated oncogenic potential and half appear to be primarily related to suppressing the activity of Src-related protein kinases and could be classified as anti-oncogenes.

While distinct in their overall molecular
10 structure, each member of a given morphotypic family of cytoplasmic protein tyrosine kinases shares sequence homology in certain non-catalytic domains in addition to sharing sequence homology in the catalytic kinase domain. Examples of defined non-catalytic
15 domains include the SH2 (SRC homology domain 2; Sadowski, I et al., Mol. Cell. Biol. 6:4396-4408; Kock, C.A. et al., 1991, Science 252:668-674) domains, SH3 domains (Mayer, B.J. et al., 1988, Nature 332:269-272) and PH domains (Musacchio et al., TIBS 18:343-348
20 (1993)). These non-catalytic domains are thought to be important in the regulation of protein-protein interactions during signal transduction (Pawson, T. and Gish, G., 1992, Cell 71:359-362).

While the metabolic roles of cytoplasmic protein
25 tyrosine kinases are less well understood than that of the receptor-type protein tyrosine kinases, significant progress has been made in elucidating some or the processes in which this class of molecules is involved. For example, members of the src family, lck
30 and fyn, have been shown to interact with CD4/CD8 and the T cell receptor complex, and are thus implicated in T cell activation, (Veillette, A. Davidson, D., 1992, TIG 8:61-66). Some cytoplasmic protein tyrosine kinases have been linked to certain phases of the cell
35 cycle (Morgan, D.O. et al., 1989, Cell 57:775-786; Kipreos, E.T. et al., 1990, Science 248:217-220;

Weaver et al., 1991, Mol. Cell. Biol. 11:4415-4422), and cytoplasmic protein tyrosine kinases have been implicated in neuronal and hematopoietic development (Maness, P., 1992, Dev. Neurosci 14:257-270 and

5 Rawlings et al., Science 261:358-361 (1993)).

Deregulation of kinase activity through mutation or overexpression is a well-established mechanism underlying cell transformation (Hunter et al., 1985, *supra*; Ullrich et al., *supra*).

10 A variety of cytoplasmic tyrosine kinases are expressed in, and may have important functions in, hematopoietic cells including *src*, *lyn*, *fyn*, *blk*, *lck*, *csk* and *hck*. (Eisenian, E. and J.B. Bolen, Cancer Cells 2(10):303-310, 1990). T-cell activation, for
15 example, is associated with activation of *lck*. The signaling activity of *lyn* may be stimulated by binding of allergens to IgE on the surface of basophils. (Eisenian, *supra*).

Abnormalities in tyrosine kinase regulated signal
20 transduction pathways can result in a number of disease states. For example, mutations in the cytoplasmic tyrosine kinase *atk* (also called *btck*) are responsible for the x-linked agammaglobulinemia, (Ventre, D., et al., Nature 361:226-23, 1993). This
25 defect appears to prevent the normal differentiation of pre-B cells to mature circulating B cells and results in a complete lack of serum immunoglobulins of all isotypes. The cytoplasmic tyrosine kinase Zap-70 has been suggested as indispensable for the
30 development of CD8 single-positive T cells as well as for signal transduction and function of single-positive CD4 T cells, and lack of this protein leads of an immunodeficiency disease in humans, (Arpala, E., et al., Cell 76:1-20, 1994). Gene knockout
35 experiments in mice suggest a role for *src* in the regulation of osteoclast function and bone remodeling

as these mice develop osteopetrosis. (Soriano et al., Cell 64:693-702, 1991 and Lowe et al., PNAS (in press)).

Megakaryocytes are large cells normally present
5 in bone marrow and spleen and are the progenitor cell
for blood platelets. Megakaryocytes are associated
with such disease states as acute megakaryocytic
leukemia (Lu et al., Cancer Genet Cytogenet, 67(2):81-
89 (1993) and Moody et al., Pediatr Radiol. 19(6-
10 7):486-488 (1989)), a disease that is difficult to
diagnose early and which is characterized by aberrant
proliferation of immature cells or "blasts";
myelofibrosis (Smith et al., Crit Rev Oncol Hematol.
10(4):305-314 (1990) and Marino, J. Am. Osteopath
15 Assoc. 10:1323-1326 (1989)), an often fatal disease
where the malignant cell may be of megakaryocytic
lineage and may be mediated by platelet or
megakaryocyte growth factors; acute megakaryocytic
myelosis (Fohlmeister et al., Haematologia 19(2):151-
20 160 (1986)) a rapidly fatal disease characterized by
megakaryocytic proliferation and the appearance of
immature megakaryocytes in the circulation; and acute
myelosclerosis (Butler et al., Cancer 49(12):2497-2499
(1982) and Bearman et al., Cancer 43(1):279-93 (1979))
25 a myeloproliferative syndrome where the marrow is
characterized by atypical megakaryocytes.

Platelets play a key role in the regulation of
blood clotting and wound healing, as well as being
associated with such disease conditions as
30 thrombocytopenia, atherosclerosis, restenosis and
leukemia. Several receptor tyrosine kinases have been
identified in human megakaryocytes including c-kit,
blg and blk. (Hoffman, H., Blood 74:1196-1212, 1989;
Long, M.W., Stem Cells 11:33-40, 1993; Zaabo, K.M., et
35 al., Cell 63:213-224, 1990). Cytoplasmic tyrosine
kinases of human megakaryocytic origin have also been

reported. (Bennett et al., Journal of Biological Chemistry 289(2):1068-1074, 1994; Lee et al., Gene 1-5, 1993; and Sakano et al., Oncogene 9:1155-1161 (1994)).

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3. Summary of the Invention

The present invention relates to novel, cytoplasmic tyrosine kinases isolated from megakaryocytes (megakaryocyte kinases or MKKs) which
10 are involved in cellular signal transduction pathways. Particular MKKs described herein are referred to as MKK1, MKK2, and MKK3. The complete nucleotide sequences encoding MKK1, MKK2, and MKK3 are disclosed herein, and provide the basis for several aspects of
15 the invention hereinafter described.

The present invention is based, in part, upon the discovery that MKK1, MKK2, and MKK3 have amino acid and structural homology, respectively, to the PTKs csk (Brauninger et al. Gene, 110:205-211 (1992) and
20 Brauninger et al., Oncogene, 8:1365-1369 (1993)), atk/btk, tec and tsk (Vetrie et al., Nature 361:226-233 (1993); Mano et al., Oncogene 8:417-424 (1993) and Heyeck et al., PNAS USA 90:669-673, 1993, respectively) and fyn (Kawakami et al. Mol. Cell. Bio. 6:4195-4201,
25 1986)).

The present invention also relates, in part, to nucleotide sequences and expression vectors encoding MKKs. Also described herein are methods of treatment and diagnosis of diseases resulting from abnormalities
30 in signal transduction pathways in which MKKs are involved.

The MKK sequences disclosed herein may be used to detect and quantify levels of MKK mRNA in cells and furthermore for diagnostic purposes for detection of
35 expression of MKKs in cells. For example, an MKK sequence may be used in hybridization assays of

biopsied tissue to diagnose abnormalities in gene expression associated with a transformed phenotype.

Also disclosed herein are methods of treatment of diseases or conditions associated with abnormalities in signal transduction pathways in megakaryocytes. Such abnormalities can result in, for example, under production of mature, differentiated cells, inappropriate proliferation of immature cells or modulation of activity of other important cellular functions.

Anti-MKK antibodies may be used for diagnostic purposes for the detection of MKKs in tissues and cells. Anti-MKK antibodies may also be used for therapeutic purposes, for example, in neutralizing the activity of an MKK associated with a signal transduction pathway.

Oligonucleotide sequences, including anti-sense RNA and DNA molecules and ribozymes, designed to inhibit the translation of MKK mRNA, may be used therapeutically in the treatment of disease states associated with aberrant expression of MKKs. In a particular embodiment of the invention described by way of Example 9 herein, an anti-MKK1 antisense molecule is used to inhibit MKK-1 protein synthesis resulting in reduced megakaryocyte growth and differentiation.

Proteins, peptides and organic molecules capable of modulating activity of MKKs may be used therapeutically in the treatment of disease states associated with aberrant expression of MKKs. Alternatively, proteins, peptides and organic molecules capable of modulating activity of MKKs may be used therapeutically to enhance normal activity levels of MKKs. For example, small molecules found to stimulate MKK1 activity in megakaryocytes may be used for ex vivo culturing of megakaryocytes intended for

autologous treatment of patients receiving chemotherapy or other therapies which deplete megakaryocytes or platelets, or in the treatment of thrombocytopenia.

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4. Brief Description of the Figures

Sub a Figures 1A and 1B. Human MKK1 nucleotide sequence (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2). Marked regions show the signal sequence, the SH2 and SH3 domains, and the catalytic domain.

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Figures 2A and 2B. Human MKK2 nucleotide sequence (SEQ ID NO:3) and deduced amino acid sequence (SEQ ID NO:4). Marked regions show the signal sequence, the pleckstrin homology domain (PH), the proline rich sequences following the PH domain, the SH2 and SH3 domains, and the catalytic domain.

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Sub a2 Figures 3A and 3B. Human MKK3 nucleotide sequence (SEQ ID NO:5) and deduced amino acid sequence (SEQ ID NO:6). Marked regions show the signal sequence, the SH2 and SH3 domains, and the catalytic domain.

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Figure 4. Expression of MKK1 and MKK2 in human and rodent cell lines.

25 Figure 5. Immunoprecipitation (i.p.) of *in vitro* transcribed and translated MKK1 and MKK2 proteins. Samples in lanes designated 1 through 9 are as follows: 1. MKK1 i.p. with anti-carboxy terminus MKK1 Ab, 2. and 3. MKK1 i.p. with anti-amino terminus MKK1 Ab, 4. MKK1 i.p. with rabbit pre immune sera, 5. MKK2 i.p. with rabbit pre immune sera, 6. and 7. MKK2 i.p. with anti-carboxy terminus MKK2 Ab, 8. MKK1 *in vitro* transcribed/translated protein without i.p., 9. MKK2 *in vitro* transcribed/translated protein without i.p.

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Figures 6A and 6B. Figures 6A-6B illustrate anti-sense MKK1 expression suppresses AChE Production in primary murine bone marrow cultures. Figure 6A illustrates AChE production. Figure 6B illustrates MKK1 protein expression.

Figure 7. MKK2 and MKK3 autophosphorylate and transphosphorylate proteins when expressed in bacteria. Lanes 2, 4, and 6 represent non-induced bacteria expressing MKK1, MKK2, MKK3, respectively. Lanes 1, 3, and 5 represent induced bacteria expressing MKK1, MKK2, MKK3, respectively.

Figure 8. MKK expression constructs.

Figure 9. Shared amino acid sequence homology of MKK1 and csk.

Figures 10A and 10B. Shared amino acid sequence homology of MKK2 and atk/btk.

Figures 11A, 11B, 11C and 11D. Shared amino acid sequence homology of MKK3 and src tyrosine kinase family members.

Figure 12. Figure 12 illustrates that the hyperexpression of MKK-1 in L-8057 cells grown in serum-free media inhibits cell growth of those cells as compared to control L-8057 cells.

Figure 13. Figure 13 illustrates the stimulation of MKK-1 infected L-8057 cells and control L-8057 cells with rat stem cell factor and IL-3.

Figure 14. Figure 14 illustrates the effect of tetradecanoyl phorbol acetate ("TPA") on either control cells or cells that express MKK-1.

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5. Detailed Description

The present invention relates to novel, cytosolic megakaryocytic kinases referred to herein as "MKKs", and in particular to megakaryocyte kinase 1 (MKK1), megakaryocyte kinase 2 (MKK2), which are expressed in

human megakaryocytic cell lines, and megakaryocyte kinase 3 (MKK3).

As used herein, MKK is a term which refers to MKK1, MKK2 and MKK3 from any species, including,
5 bovine, ovine, porcine, equine, murine and preferably human, in naturally occurring-sequence or in variant form, or from any source, whether natural, synthetic, or recombinant. A preferred MKK variant is one having at least 80% amino acid homology, a particularly
10 preferred MKK variant is one having at least 90% sequence homology and another particularly preferred MKK variant is one having at least 95% amino acid homology to the naturally occurring MKK.

MKK1 is a cytosolic tyrosine kinase of molecular
15 weight 58 kD, as determined by SDS gel electrophoresis, having homology to the TK csk (Partanen, et al., Oncogene 6:2013-2018 (1991) and Nada et al., Nature 351:69-72 (1991)) in the intervening sequences of its catalytic domain, the SH2
20 and SH3 domains, and other non-catalytic regions and like csk, lacks regulatory phosphorylation sites corresponding to c-src tyrosines 416 and 527. MKK1 also lacks an amino-terminal myristylation site.

Csk is a recently described novel cytoplasmic TK
25 that seems to play a key role in regulation of signal transduction in hematopoietic and neural development. For example csk has been shown to negatively regulate members of the src family of TKs, including c-src, lck, and fyn, through its ability to phosphorylate
30 regulatory tyrosines. (Bergman et al., The EMBO Journal 11(8):2919-2924 (1992) and Sabe et al., Molecular and Cellular Biology 12(10):4706-4713 (1992)). Autero et al., (Molecular and Cellular Biology 14(2):1308-1321 (1994)) have reported that csk
35 positively regulates a phosphatase, CD45, that is key to T-cell activity. Csk mediated phosphorylation of

CD45 phosphotyrosine phosphatase (PTPase) caused a several fold increase in its PTPase activity. Csk appears to play a role as a regulator of the sequence of both phosphorylation and dephosphorylation events
5 culminating in cell activation and proliferation.

Defective expression of csk in mouse embryos results in defects in the neural tube with subsequent death between day 9 and day 10 of gestation, with cells derived from these embryos exhibiting an order
10 of magnitude increase in activity of src kinase (Nada et al., Cell 73:1125-1135 (1993)). Overexpression of csk in transformed rat 3Y1 fibroblasts was shown to cause reversion to normal phenotypes (Sabe et al., Molecular and Cellular Biology 12:4706-4713 (1992)).

15 MKK1 has 54% homology with csk at the amino acid level and structural similarity to csk, i.e., the lack of regulatory phosphorylation sites and the lack of an amino-terminal myristylation site. Experimental data, see Section 9, show that expression of human anti-
20 sense MKK1 sequences inhibits synthesis of murine MKK1, which inhibition is associated with a reduction of proliferation of megakaryocytes *in vitro*. Based upon the experimental data in Section 9 and amino acid and structural homology with csk, MKK1 appears to play
25 a regulatory role in the growth and differentiation of megakaryocytes and perhaps neural tissues based on its expression in those tissues.

MKK2 is a novel cytosolic tyrosine kinase of molecular weight 78kD, as determined by SDS gel
30 electrophoresis, having homology to the tec subfamily of TKs which also includes tsk and atk/btk. Like the tec subfamily, MKK2 lacks an amino-terminal site for myristylation and has a putative pleckstrin homology binding domain located 5' to the SH3 domain (Musacchio
35 et al., TIBS 18:343-348 (1993)). The pleckstrin homology (PH) domain has been found in a number of

proteins with diverse cellular functions and is abundant in proteins involved in signal transduction pathways. Musacchio et al., *supra* suggest that the PH domain may be involved in molecular recognition
5 similarly to SH2 and SH3 domains.

The tec family of tyrosine kinases appear to play roles in cellular differentiation and include family members tec, a kinase which may be specifically involved in the cell growth of hepatocytes or
10 hepatocarcinogenesis (Mano et al., *supra*); tsk, which may play a role in early T-lymphocyte differentiation (Heyek et al., PNAS USA 90:669-673 (1993)) and atk/btk. Aberrant expression of atk/btk has been shown to be responsible for X-linked
15 agammaglobulinemia (XLA), a human disease resulting from a developmental block in the transition from pre-B cells to mature B cells (Ventre, D. et al., *supra*).

MKK2 has 50% homology to atk/btk at the amino acid level and structural similarity to tec family
20 members, i.e., the presence of the SH2, SH3 and PH domains and the lack of an amino-terminal site for myristylation and the carboxyl site of tyrosine phosphorylation found in family members. Based upon the amino acid homology and structural similarity to
25 tec family members which play roles in cellular differentiation, MKK2 may play a role in the differentiation of megakaryocytes.

MKK3 is a novel cytosolic tyrosine kinase of molecular weight 58kD, as determined by SDS gel
30 electrophoresis, having homology to the TK fyn. MKK3 does not have a myristylation sites. MKK3 does have a putative regulatory cite at tyr 387 but the surrounding 12 amino acids are not identical with other members of the src subfamily that share highly
35 conserved sequences in this region. MKK3 has 47% homology with fyn at the amino acid level.

The fyn gene was originally characterized in normal human fibroblast and endothelial cells, but it is also expressed in a variety of other cell types. Alternative splicing of fyn has been shown to yield
5 two distinct transcripts, both coding for enzymatically active forms of the kinases.

MKK sequences could be used diagnostically to measure expression of MKKs in disease states, such as for example leukemia, where abnormal proliferation of
10 immature myeloid cells occurs, or where abnormal differentiation of megakaryocytes occurs. MKKs could also be used therapeutically in the treatment of disease states involving abnormal proliferation or differentiation through interruption of signal
15 transduction by modulation of protein tyrosine kinases.

See 95 The nucleotide and deduced amino acid sequence of human MKK1, MKK2, and MKK3 are shown in Figures 1A-1B, 2A-2B and 3A-3B, respectively. Figures 9, 10A-10B and
20 11A-11D show the shared sequence homology between MKKs and related tyrosine kinases.

5.1. The MKK Coding Sequences

The nucleotide coding sequence and deduced amino
25 acid sequence of the human MKK1, MKK2, and MKK3 genes are depicted in Figures 1A-1B, 2A-2B and 3A-3B, respectively. In accordance with the invention, any nucleotide sequence which encodes the amino acid sequence of an MKK gene product can be used to
30 generate recombinant molecules which direct the expression of an MKK.

See 95 In a specific embodiment described herein, the human MKK1, MKK2, and MKK3 genes were isolated by performing polymerase chain reactions (PCR) in
35 combination with two degenerate oligonucleotide primer pools that were designed on the basis of highly

conserved sequences within the kinase domain of
receptor tyrosine kinases corresponding to the amino
acid sequence HRDLAA (sense primer) and SDVWS/FY
(antisense primer) (Hanks et al., 1988). The MKK
5 cDNAs were synthesized by reverse transcription of
poly-A RNA from the human K-562 cell line, ATCC
accession number CCL 243, or from the Meg 01 cell
line, (Ogura et al., Blood 66:1384 (1985)).

Sula 7 The PCR fragments were used to screen a lambda
10 gt11 library of human fetal brain. For each
individual MKK, several overlapping clones were
identified. The composite of the cDNA clones for
MKK1, MKK2, and MKK3 are depicted in Figures 1A-1B,
2A-2B, and 3A-3B, respectively.

15 Further characterization of the individual MKKs
is found *infra*.

5.2. Expression of MKK

In accordance with the invention, MKK
20 polynucleotide sequences which encode MKKs, peptide
fragments of MKKs, MKK fusion proteins or functional
equivalents thereof, may be used to generate
recombinant DNA molecules that direct the expression
of MKK protein, MKK peptide fragment, fusion proteins
25 or a functional equivalent thereof, in appropriate
host cells. Such MKK polynucleotide sequences, as
well as other polynucleotides which selectively
hybridize to at least a part of such MKK
polynucleotides or their complements, may also be used
30 in nucleic acid hybridization assays, Southern and
Northern blot analyses, etc.

Due to the inherent degeneracy of the genetic
code, other DNA sequences which encode substantially
the same or a functionally equivalent amino acid
35 sequence, may be used in the practice of the invention
for the cloning and expression of the MKK protein.

Such DNA sequences include those which are capable of hybridizing to the human MKK sequence under stringent conditions. The phrase "stringent conditions" as used herein refers to those hybridizing conditions that (1) 5 employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0.1% SDS at 50°C.; (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine 10 serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C; or (3) employ 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M Sodium pyrophosphate, 5 x Denhardt's solution, sonicated 15 salmon sperm DNA (50 g/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC and 0.1% SDS.

Altered DNA sequences which may be used in accordance with the invention include deletions, additions 20 or substitutions of different nucleotide residues resulting in a sequence that encodes the same or a functionally equivalent gene product. The gene product itself may contain deletions, additions or substitutions of amino acid residues within an MKK 25 sequence, which result in a silent change thus producing a functionally equivalent MKK. Such amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipatic nature of the 30 residues involved. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; amino acids with uncharged polar head groups having similar hydrophilicity values include the 35 following: leucine, isoleucine, valine; glycine,

alanine; asparagine, glutamine; serine, threonine; phenylalanine, tyrosine.

The DNA sequences of the invention may be engineered in order to alter an MKK coding sequence for a variety of ends including but not limited to alterations which modify processing and expression of the gene product. For example, mutations may be introduced using techniques which are well known in the art, e.g., site-directed mutagenesis, to insert new restriction sites, to alter glycosylation patterns, phosphorylation, etc.

In another embodiment of the invention, an MKK or a modified MKK sequence may be ligated to a heterologous sequence to encode a fusion protein. For example, for screening of peptide libraries for inhibitors of MKK activity, it may be useful to encode a chimeric MKK protein expressing a heterologous epitope that is recognized by a commercially available antibody. A fusion protein may also be engineered to contain a cleavage site located between an MKK sequence and the heterologous protein sequence, so that the MKK may be cleaved away from the heterologous moiety.

In an alternate embodiment of the invention, the coding sequence of an MKK could be synthesized in whole or in part, using chemical methods well known in the art. See, for example, Caruthers et al., 1980, Nuc. Acids Res. Symp. Ser. 7:215-233; Crea and Horn, 180, Nuc. Acids Res. 9(10):2331; Matteucci and Caruthers, 1980, Tetrahedron Letters 21:719; and Chow and Kempe, 1981, Nuc. Acids Res. 9(12):2807-2817. Alternatively, the protein itself could be produced using chemical methods to synthesize an MKK amino acid sequence in whole or in part. For example, peptides can be synthesized by solid phase techniques, cleaved from the resin, and purified by preparative high

performance liquid chromatography. (e.g., see Creighton, 1983, Proteins Structures And Molecular Principles, W.H. Freeman and Co., N.Y. pp. 50-60).

The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure; see Creighton, 1983, Proteins, Structures and Molecular Principles, W.H. Freeman and Co., N.Y., pp. 34-49.

In order to express a biologically active MKK, the nucleotide sequence coding for MKK, or a functional equivalent, is inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. The MKK gene products as well as host cells or cell lines transfected or transformed with recombinant MKK expression vectors can be used for a variety of purposes. These include but are not limited to generating antibodies (i.e., monoclonal or polyclonal) that competitively inhibit activity of an MKK and neutralize its activity. Anti-MKK antibodies may be used in detecting and quantifying expression of an MKK in cells and tissues.

5.3. Expression Systems

Methods which are well known to those skilled in the art can be used to construct expression vectors containing an MKK coding sequence and appropriate transcriptional/translational control signals. These methods include *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* recombination/genetic recombination. See, for example, the techniques described in Maniatis et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current Protocols in

Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y.

A variety of host-expression vector systems may be utilized to express an MKK coding sequence. These
5 include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing an MKK coding sequence; yeast transformed with recombinant yeast expression vectors containing
10 an MKK coding sequence; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing an MKK coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus,
15 CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing an MKK coding sequence; or animal cell systems. The expression elements of these systems vary in their strength and specificities.
20 Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used in the expression vector. For example, when cloning in bacterial systems, inducible
25 promoters such as pL of bacteriophage λ , plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used; when cloning in insect cell systems, promoters such as the baculovirus polyhedrin promoter may be used; when cloning in plant cell systems, promoters
30 derived from the genome of plant cells (e.g., heat shock promoters; the promoter for the small subunit of RUBISCO; the promoter for the chlorophyll a/b binding protein) or from plant viruses (e.g., the 35S RNA promoter of CaMV; the coat protein promoter of TMV)
35 may be used; when cloning in mammalian cell systems, promoters derived from the genome of mammalian cells

(e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5 K promoter) may be used; when
5 an MKK DNA, SV40-, BPV- and EBV-based vectors may be used with an appropriate selectable marker.

In bacterial systems a number of expression vectors may be advantageously selected depending upon the use intended for the MKK expressed. For example,
10 when large quantities of MKK1 are to be produced for the generation of antibodies, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include but are not limited to the E. coli
15 expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which the MKK1 coding sequence may be ligated into the vector in frame with the lac Z coding region so that a hybrid AS-lac Z protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic acids Res.
20 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can
25 easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned polypeptide of
30 interest can be released from the GST moiety.

In yeast, a number of vectors containing constitutive or inducible promoters may be used. For a review see, Current Protocols in Molecular Biology, Vol. 2, 1988, Ed. Ausubel et al., Greene Publish.
35 Assoc. & Wiley Interscience, Ch. 13; Grant et al., 1987, Expression and Secretion Vectors for Yeast, in

Methods in Enzymology, Ed. Wu & Grossman, 1987, Acad. Press, N.Y. 153:516-544; Glover, 1986, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3; and Bitter, 1987, Heterologous Gene Expression in Yeast, Methods
5 in Enzymology, Eds. Berger & Kimmel, Acad. Press, N.Y. 152:673-684; and The Molecular Biology of the Yeast Saccharomyces, 1982, Eds. Strathern et al., Cold Spring Harbor Press, Vols. I and II.

In cases where plant expression vectors are used,
10 the expression of an MKK coding sequence may be driven by any of a number of promoters. For example, viral promoters such as the 35S RNA and 19S RNA promoters of CaMV (Brisson et al., 1984, Nature 310:511-514), or the coat protein promoter of TMV (Takamatsu et al.,
15 1987, EMBO J. 6:307-311) may be used; alternatively, plant promoters such as the small subunit of RUBISCO (Coruzzi et al., 1984, EMBO J. 3:1671-1680; Broglie et al., 1984, Science 224:838-843); or heat shock
20 promoters, e.g., soybean hsp17.5-E or hsp17.3-B (Gurley et al., 1986, Mol. Cell. Biol. 6:559-565) may be used. These constructs can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation,
25 microinjection, electroporation, etc. For reviews of such techniques see, for example, Weissbach & Weissbach, 1988, Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp. 421-463; and Grierson & Corey, 1988, Plant Molecular Biology, 2d Ed., Blackie, London, Ch. 7-9.

30 An alternative expression system which could be used to express an MKK is an insect system. In one such system, Autographa californica nuclear polyhydrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in Spodoptera
35 frugiperda cells. An MKK coding sequence may be cloned into non-essential regions (for example the

polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example, the polyhedrin promoter). Successful insertion of an MKK coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect Spodoptera frugiperda cells in which the inserted gene is expressed. (e.g., see Smith et al., 1983, J. Virol. 46:584; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, an MKK coding sequence may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing an MKK in infected hosts. (e.g., See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. (USA) 81:3655-3659).

Alternatively, the vaccinia 7.5 K promoter may be used. (See, e.g., Mackett et al., 1982, Proc. Natl. Acad. Sci. (USA) 79:7415-7419; Mackett et al., 1984, J. Virol. 49:857-864; Panicali et al., 1982, Proc. Natl. Acad. Sci. 79:4927-4931).

Specific initiation signals may also be required for efficient translation of an inserted MKK coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire MKK gene, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational

control signals may be needed. However, in cases where only a portion of an MKK coding sequence is inserted, exogenous translational control signals, including the ATG initiation codon, must be provided.

5 Furthermore, the initiation codon must be in phase with the reading frame of an MKK coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural
10 and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al., 1987, Methods in Enzymol. 153:516-544).

15 In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage)
20 of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cells lines or host systems can be chosen
25 to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the
30 gene product may be used. Such mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, WI38, etc.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For
35 example, cell lines which stably express an MKK may be engineered. Rather than using expression vectors

which contain viral origins of replication, host cells can be transformed with MKK DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express an MKK.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy et al., 1980, Cell 22:817) genes can be employed in tk⁻, hgp^rt⁻ or ap^rt⁻ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981), Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin et al., 1981, J. Mol. Biol. 150:1); and hyg^r, which confers resistance to hygromycin (Santerre et al., 1984, Gene 30:147). Recently, additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which

allows cells to utilize histinol in place of histidine (Hartman & Mulligan, 1988, Proc. Natl. Acad. Sci. USA 85:8047); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue L., 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory, Ed.).

10 **5.4. Identification of Transfectants or Transformants that Express the MKK**

 The host cells which contain the coding sequence and which express the biologically active gene product may be identified by at least four general approaches; (a) DNA-DNA or DNA-RNA hybridization; (b) the presence or absence of "marker" gene functions; (c) assessing the level of transcription as measured by the expression of MKK mRNA transcripts in the host cell; and (d) detection of the gene product as measured by immunoassay or by its biological activity.

20 In the first approach, the presence of the MKK coding sequence inserted in the expression vector can be detected by DNA-DNA or DNA-RNA hybridization using probes comprising nucleotide sequences that are homologous to the MKK coding sequence, respectively, or portions or derivatives thereof.

25 In the second approach, the recombinant expression vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, resistance to methotrexate, transformation phenotype, occlusion body formation in baculovirus, etc.). For example, if the MKK1 coding sequence is inserted within a marker gene sequence of the vector, recombinant cells containing the MKK1 coding sequence can be identified by the absence of

the marker gene function. Alternatively, a marker gene can be placed in tandem with an MKK sequence under the control of the same or different promoter used to control the expression of the MKK coding
5 sequence. Expression of the marker in response to induction or selection indicates expression of the MKK coding sequence.

In the third approach, transcriptional activity for an MKK coding region can be assessed by
10 hybridization assays. For example, RNA can be isolated and analyzed by Northern blot using a probe homologous to an MKK coding sequence or particular portions thereof. Alternatively, total nucleic acids of the host cell may be extracted and assayed for
15 hybridization to such probes.

In the fourth approach, the expression of an MKK protein product can be assessed immunologically, for example by Western blots, immunoassays such as radioimmuno-precipitation, enzyme-linked immunoassays
20 and the like.

5.5. Uses of MKK and Engineered Cell Lines

Megakaryocytes, the progenitor cell for blood platelets, and platelets are associated with disease
25 states involving aberrant proliferation or differentiation of such cells, such as acute megakaryocytic leukemia, acute megakaryocytic myelosis and thrombocytopenia. MKKs appear to play a role in the growth and differentiation of megakaryocytes,
30 therefore inhibitors of MKKs may be used therapeutically for the treatment of diseases states resulting from aberrant growth of megakaryocytes or platelets. Alternatively, enhancers of MKKs may be used therapeutically to stimulate the proliferation of
35 megakaryocytes in such applications as, for example, ex vivo culturing of megakaryocytes intended for

autologous cell therapy in individuals receiving chemotherapy or other therapies which deplete megakaryocytes or platelets or in treating thrombocytopenia caused by other conditions.

- 5 In an embodiment of the invention, an MKK and/or cell line that expresses an MKK may be used to screen for antibodies, peptides, or other molecules that act as agonists or antagonists of MKK through modulation of signal transduction pathways. For example, anti-
- 10 MKK antibodies capable of neutralizing the activity of MKK may be used to inhibit an MKK associated signal transduction pathway. Such antibodies can act intracellularly utilizing the techniques described in Marasco et al. (PNAS 90:7889-7893 (1993) for example or
- 15 through delivery by liposomes. Alternatively, screening of organic or peptide libraries with recombinantly expressed MKK protein or cell lines expressing MKK protein may be useful for identification of therapeutic molecules that function
- 20 by modulating the kinase activity of MKK or its associated signal transduction pathway. A therapeutic molecule may find application in a disease state associated with megakaryocytes, such as acute megakaryocytic leukemia, or alternatively, in non-
- 25 disease applications, for example in ex vivo culturing of megakaryocytes intended for autologous treatment of individuals undergoing chemotherapy. Synthetic compounds, natural products, and other sources of potentially biologically active materials can be
- 30 screened in a number of ways deemed to be routine to those of skill in the art.

- The ability of antibodies, peptides, or other molecules to prevent or mimic, the effect of MKK on signal transduction responses on MKK expressing cells
- 35 may be measured. For example, responses such as activation or inhibition of MKK kinase activity or

modulation of second messenger production may be monitored. The term "second messenger" as used herein refers to any component or product found in the cascade of signal transduction events. These assays
5 may be performed using conventional techniques developed for these purposes.

5.5.1. Antibody Production and Screening

10 Various procedures known in the art may be used for the production of antibodies to epitopes of the recombinantly produced MKK. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments and fragments
15 produced by a Fab expression library. Neutralizing antibodies, i.e., those which inhibit the biological activity, i.e., the kinase activity, of an MKK are especially preferred for diagnostics and therapeutics.

For the production of antibodies, various host
20 animals may be immunized by injection with an MKK protein including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's
25 (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants
30 such as BCG (bacilli Calmette-Guerin) and Corynebacterium parvum.

Monoclonal antibodies to an MKK may be prepared by using any technique which provides for the production of antibody molecules by continuous cell
35 lines in culture. These include but are not limited to the hybridoma technique originally described by

- Koehler and Milstein, (Nature, 1975, 256:495-497), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today, 4:72; Cote et al., 1983, Proc. Natl. Acad. Sci., 80:2026-2030) and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci. 81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985, Nature 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used.
- 15 Alternatively, techniques described for the production of single chain antibodies (U.S. Patent No. 4,946,778) can be adapted to produce an MKK-specific single chain antibodies.

- Antibody fragments which contain specific binding sites of an MKK may be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')₂ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')₂ fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity the MKK of interest.

5.5.2. Screening of Peptide Library with MKK or MKK Engineered Cell Lines

- Random peptide libraries consisting of all possible combinations of amino acids attached to a

solid phase support may be used to identify peptides that are able to bind to MKK binding sites, e.g., SH2, SH3 or PH binding sites, or other functional domains of an MKK, such as kinase domains. The screening of
5 peptide libraries may have therapeutic value in the discovery of pharmaceutical agents that act to stimulate or inhibit the biological activity of an MKK.

Identification of molecules that are able to bind
10 to an MKK may be accomplished by screening a peptide library with recombinant MKK protein. Methods for expression of an MKK are described in Section 5.2, 5.3 and 5.4 and may be used to express a recombinant full length MKK or fragments of an MKK depending on the
15 functional domains of interest. For example, the kinase and SH2, SH3 or PH binding domains of an MKK may be separately expressed and used to screen peptide libraries.

To identify and isolate the peptide/solid phase
20 support that interacts and forms a complex with an MKK, it is necessary to label or "tag" the MKK molecule. The MKK protein may be conjugated to enzymes such as alkaline phosphatase or horseradish peroxidase or to other reagents such as fluorescent
25 labels which may include fluorescein isothiocyanate (FITC), phycoerythrin (PE) or rhodamine. Conjugation of any given label to MKK may be performed using techniques that are routine in the art.

Alternatively, MKK expression vectors may be
30 engineered to express a chimeric MKK protein containing an epitope for which a commercially available antibody exists. The epitope specific antibody may be tagged using methods well known in the art including labeling with enzymes, fluorescent dyes
35 or colored or magnetic beads.

The "tagged" MKK conjugate is incubated with the random peptide library for 30 minutes to one hour at 22°C to allow complex formation between an MKK and peptide species within the library. The library is
5 then washed to remove any unbound MKK protein. If MKK has been conjugated to alkaline phosphatase or horseradish peroxidase the whole library is poured into a petri dish containing a substrates for either alkaline phosphatase or peroxidase, for example, 5-
10 bromo-4-chloro-3-indoyl phosphate (BCIP) or 3,3',4,4"-diaminobenzidine (DAB), respectively. After incubating for several minutes, the peptide/solid phase-MKK complex changes color, and can be easily identified and isolated physically under a dissecting
15 microscope with a micromanipulator. If a fluorescent tagged MKK molecule has been used, complexes may be isolated by fluorescent activated sorting. If a chimeric MKK protein expressing a heterologous epitope has been used, detection of the peptide/MKK complex
20 may be accomplished by using a labeled epitope specific antibody. Once isolated, the identity of the peptide attached to the solid phase support may be determined by peptide sequencing.

25 **5.5.3. Screening of Organic
 Compounds with MKK Protein or
 Engineered Cell Lines**

Cell lines that express an MKK may be used to screen for molecules that modulate MKK activity or signal transduction. Such molecules may include small
30 organic or inorganic compounds or extracts of biological materials such as plants, fungi, etc., or other molecules that modulate MKK activity or that promote or prevent MKK mediated signal transduction. Synthetic compounds, natural products, and other
35

sources of potentially biologically active materials can be screened in a number of ways.

The ability of a test molecule to interfere with MKK signal transduction may be measured using standard
5 biochemical techniques. Other responses such as activation or suppression of catalytic activity, phosphorylation or dephosphorylation of other proteins, activation or modulation of second messenger production, changes in cellular ion levels,
10 association, dissociation or translocation of signalling molecules, or transcription or translation of specific genes may also be monitored. These assays may be performed using conventional techniques developed for these purposes in the course of
15 screening. (See, for example, Peralidi, et al., J. Biochem. 285:71-78 (1992) or Campbell et al., JBC 268:7427-7434 (1993)).

Cellular processes under the control of an MKK signalling pathway may include, but are not limited
20 to, normal cellular functions such as proliferation or differentiation of megakaryocytes or platelets, in addition to abnormal or potentially deleterious processes such as unregulated or inappropriate cell proliferation, blocking of differentiation of
25 megakaryocytes or platelets, or ultimately cell death. The qualitative or quantitative observation and measurement of any of the described cellular processes by techniques known in the art may be advantageously used as a means of scoring for signal transduction in
30 the course of screening.

MKK, or functional derivatives thereof, useful in identifying compounds capable of modulating signal transduction may have, for example, amino acid deletions and/or insertions and/or substitutions as
35 long as they retain significant ability to interact with some or all relevant components of a MKK signal

transduction pathway. A functional derivative of MKK may be prepared from a naturally occurring or recombinantly expressed MKK by proteolytic cleavage followed by conventional purification procedures known to those skilled in the art. Alternatively, the functional derivative may be produced by recombinant DNA technology by expressing parts of MKK which include the functional domain in suitable cells. Functional derivatives may also be chemically synthesized. Cells expressing MKK may be used as a source of MKK, crude or purified for testing in these assays.

MKK signal transduction activity may be measured by standard biochemical techniques or by monitoring the cellular processes controlled by the signal. To assess modulation of kinase activity, the test molecule is added to a reaction mixture containing MKK and a substrate. The kinase reaction is then initiated with the addition of ATP. An immunoassay using an antiphosphotyrosine antibody is performed on the kinase reaction to detect the presence or absence of the phosphorylated tyrosine residues on the substrate or to detect phosphorylated tyrosine residues on autophosphorylated MKK, and results are compared to those obtained for controls *i.e.*, reaction mixtures not exposed to the test molecule.

5.6. Uses of MKK Polynucleotide

An MKK polynucleotide may be used for diagnostic and/or therapeutic purposes. For diagnostic purposes, an MKK polynucleotide may be used to detect MKK gene expression or aberrant MKK gene expression in disease states, *e.g.*, acute megakaryocytic leukemia or acute megakaryocytic myelosis. Included in the scope of the invention are oligonucleotide sequences, that include antisense RNA and DNA molecules and ribozymes, that

function to inhibit translation of an MKK. In a specific embodiment of this aspect of the invention, an anti-MKK1 antisense molecule is shown to inhibit MKK-1 protein synthesis resulting in reduced
5 megakaryocyte growth and differentiation.

5.6.1. Diagnostic Uses of an MKK Polynucleotide

10 An MKK polynucleotide may have a number of uses for the diagnosis of diseases resulting from aberrant expression of MKK. For example, the MKK1 DNA sequence may be used in hybridization assays of biopsies or autopsies to diagnose abnormalities of MKK1
15 expression; e.g., Southern or Northern analysis, including *in situ* hybridization assays. Such techniques are well known in the art, and are in fact the basis of many commercially available diagnostic kits.

20 5.6.2. Therapeutic Uses of an MKK Polynucleotide

An MKK polynucleotide may be useful in the treatment of various abnormal conditions. By introducing gene sequences into cells, gene therapy
25 can be used to treat conditions in which the cells do not proliferate or differentiate normally due to underexpression of normal MKK or expression of abnormal/inactive MKK. In some instances, the polynucleotide encoding an MKK is intended to replace or act in the place of a functionally deficient
30 endogenous gene. Alternatively, abnormal conditions characterized by overproliferation can be treated using the gene therapy techniques described below.

Abnormal proliferation of megakaryocytes is an
35 important component of a variety of disease states such as acute megakaryocytic leukemia, myelofibrosis,

or acute megakaryocytic myelosis. Recombinant gene therapy vectors, such as viral vectors, may be engineered to express variant, signalling incompetent forms of MKK which may be used to inhibit the activity
5 of the naturally occurring endogenous MKK. A signalling incompetent form may be, for example, a truncated form of the protein that is lacking all or part of its catalytic domain. Such a truncated form may participate in normal binding to a substrate but
10 lack enzymatic activity. Thus recombinant gene therapy vectors may be used therapeutically for treatment of diseases resulting from aberrant expression or activity of an MKK. Accordingly, the invention provides a method of inhibiting the effects
15 of signal transduction by an endogenous MKK protein in a cell comprising delivering a DNA molecule encoding a signalling incompetent form of the MKK protein to the cell so that the signalling incompetent MKK protein is produced in the cell and competes with the endogenous
20 MKK protein for access to molecules in the MKK protein signalling pathway which activate or are activated by the endogenous MKK protein.

Expression vectors derived from viruses such as retroviruses, vaccinia virus, adeno-associated virus,
25 herpes viruses, or bovine papilloma virus, may be used for delivery of recombinant MKK into the targeted cell population. Methods which are well known to those skilled in the art can be used to construct recombinant viral vectors containing an MKK
30 polynucleotide sequence. See, for example, the techniques described in Maniatis et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene
35 Publishing Associates and Wiley Interscience, N.Y. Alternatively, recombinant MKK molecules can be

reconstituted into liposomes for delivery to target cells.

Oligonucleotide sequences, that include anti-sense RNA and DNA molecules and ribozymes that
5 function to inhibit the translation of an MKK mRNA are within the scope of the invention. Anti-sense RNA and DNA molecules act to directly block the translation of mRNA by binding to targeted mRNA and preventing protein translation. In regard to antisense DNA,
10 oligodeoxyribonucleotides derived from the translation initiation site, e.g., between -10 and +10 regions of an MKK nucleotide sequence, are preferred.

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific
15 hybridization of the ribozyme molecule to complementary target RNA, followed by a endonucleolytic cleavage. Within the scope of the invention are engineered hammerhead motif ribozyme molecules that specifically
20 and efficiently catalyze endonucleolytic cleavage of MKK1 RNA sequences.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage
25 sites which include the following sequences, GUA, GUU and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for predicted structural features
30 such as secondary structure that may render the oligonucleotide sequence unsuitable. The suitability of candidate targets may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease protection
35 assays.

Both anti-sense RNA and DNA molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Various modifications to the DNA molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribo- or deoxy- nucleotides to the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone.

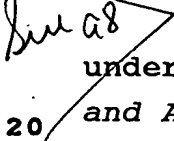
Methods for introducing polynucleotides into such cells or tissue include methods for *in vitro* introduction of polynucleotides such as the insertion of naked polynucleotide, *i.e.*, by injection into tissue, the introduction of an MKK polynucleotide in a cell *ex vivo*, *i.e.*, for use in autologous cell therapy, the use of a vector such as a virus, retrovirus, phage or plasmic, etc. or techniques such as electroporation which may be used *in vivo* or *ex vivo*.

6. Examples: Cloning and Characterization of MKK1

For clarity of discussion, the subsection below describes the isolation and characterization of a cDNA clone encoding the novel tyrosine kinase designated MKK1. The MKK2 and MKK3 genes were cloned and characterized using the same methods.

6.1. cDNA Cloning, MKK Expression and MKK Characterization

Confluent plates of K-562 cells (ATCC accession number CCL 243) were lysed by treatment with guanidinium-thiocyanate according to Chirgwin et al. (1979, Biochemistry 18:5294-5299). Total RNA was isolated by CsCl-gradient centrifugation. First-strand cDNA was synthesized from 20 µg total RNA with avian myeloblastosis virus (AMV) reverse transcriptase (Boehringer Mannheim).

Handwritten: *See 28*  cDNA was used in a polymerase chain reaction under standard conditions (*PCR Technology-Principles and Applications for DNA Amplifications*, H.E. Erlich, Ed., Stockton Press, New York 1989). Degenerate pools of primers corresponding to the amino acid sequence HRDLAA and SDVWSF/Y were prepared and used for the amplification:

5' oligo pool

		H	R	D	L	A	A	
5'	GGAATTCC	CAC	AGN	GAC	TTN	GCN	GCN	AG 3'
		T	C	A	T	C	A	C

3' oligo pool

		F/Y	S	W	V	D	S	
5'	GGAATTCC	GAA	NGT	CCA	NAC	GTC	NGA	3'
		ATG	CA			C	C	

Thirty-five PCR cycles were carried out using 8 µg (0.8 µg) of the pooled primers. (Annealing 55°C, 1 min; Extension 72°C, 2 min; Denaturation 94°C, 1 min).

The reaction product was subjected to polyacrylamide gel electrophoresis. Fragments of the expected size (~210 bp) were isolated, digested with the restriction enzyme EcoRI, and subcloned into the pBluskript vector (Stratagene) using standard techniques (*Current Protocols in Molecular Biology*, eds. F.M. Ausubel et al., John Wiley & Sons, New York, 1988).

The recombinant plasmids were transformed into the competent E. coli strain designated 298.

The subcloned PCR products were sequenced by the method of Sanger et al. (Proc. Natl. Acad. Sci. USA 74, 5463-5467) using Sequenase (United States Biochemical, Cleveland, Ohio 44111 USA). Clones designated MKK1, MKK2, and MKK3 were identified as novel TKs.

6.1.1. Full-length cDNA Cloning

Sub a9 The partial cDNA sequence of the new MKK1 TK, which was identified by PCR, was used to screen a λ gt11 library from human fetal brain cDNA (Clontech) (complexity of 1×10^{10} recombinant phages). One million independent phage clones were plated and transferred to nitrocellulose filters following standard procedures (Sambrook, H.J., Molecular Cloning, Cold Spring Harbor Laboratory Press, USA, 1989). The filters were hybridized to the EcoRI/EcoRI fragment of clone MKK1, which had been radioactively labeled using 50 μ Ci [α^{32} P]ATP and the random-primed DNA labeling kit (Boehringer Mannheim). The longest cDNA insert of ~3500 bp was digested with the restriction enzymes EcoRI/SacI to obtain a 5' end probe of 250 bp. This probe was used to rescreen the human fetal brain library and several overlapping clones were isolated. The composite of the cDNA clones of MKK1, MKK2 and MKK3 is shown in Figures 1A-1B, 2A-2B and 3A-3B, respectively. The 1.75 million independent phage

clones of a human placenta library, λ ZAP, were plated and screened with the 5' end probe (EcoRI/SacI) of the clone used above. Subcloning of positive bacteriophages clones into pBluskript vector was done
5 by the *in vivo* excision protocol (Stratagene).

See 10 The composite cDNA sequence and the predicted amino acid sequence of MKK1, MKK2 and MKK3 are shown in Figures 1A-1B, 2A-2B and 3A-3B, respectively.

10 6.1.2. MKK Expression

E.coli expression constructs for MKK1, MKK2 and MKK3 were produced by cloning of the corresponding cDNA fragments into a plasmid expression vector pTZS2 (Ray et al., PNAS USA 89:(13):5705-5709 (1992)) by
15 substitution of recoverin coding sequence with synthetic polylinker fragment. To provide in-frame connection of the coding sequences to prokaryotic translation initiation site coded by the vector, an NdeI restriction site overlapping start codon (CATATG)
20 was introduced in all three MKK cDNAs by site directed mutagenesis. The resulting constructs are designed to drive expression of unfused proteins with authentic amino acid sequences. Figure 8 shows MKK expression constructs.

25

6.1.3. RNA Blot Analysis of MKKs

Total RNA was isolated from human megakaryocytes, myeloid cells, B-cells, T-cells, and epithelial cells.

PolyA⁺ RNA was isolated on an oligo (dT) column
30 (Aviv and Leder, 1972, Proc. Natl. Acad. Sci. USA 69, 1408-1412). The poly A⁺ RNA was isolated using RNA stat -60 method (Tel-Test B Inc.) and blotted on a nitrocellulose filter using a slot blot apparatus (Schleicher and Schuell). 2 μ g of poly A⁺ RNA was
35 loaded per lane. The filter was hybridized with a ³²P-labeled EcoRI/EcoRI DNA fragment obtained by PCR.

Subsequently, the filter was exposed to x-ray film at -70°C with an intensifying screen. The results, as shown in Figure 4, suggest that MKK1 and MKK2 are preferentially expressed in megakaryocytes. MKK3 expression could not be detected using this technique. Figure 8 shows MKK expression constructs.

7. Example: Autophosphorylation of MKK2 and MKK3

Figure 7 represents Western blot analysis of protein from bacteria expressing MKK1, MKK2, or MKK3 using an anti-phosphotyrosine antibody (Hansen et al., Electrophoresis 14:112-126 (1993)). All MKK constructs were cloned into the inducible vector pTZS2, and transformed bacteria were grown under induced and uninduced conditions as described by Ray, et al., (PNAS USA 89:5705-5709 (1992)). Bacterial pellets from these cultures were resuspended in sample buffer, containing 2-mercaptoethanol and SDS, and boiled. Proteins were separated by SDS-polyacrylamide gel electrophoresis. The results of this example indicate that MKK2 and MKK3 have kinase activity.

8. Example: Production of Anti-MKK Antibodies and Immunoprecipitation of MKK

Subsa Antibodies recognizing MKK1 and MKK2 protein were made in rabbits using standard procedures. The anti-carboxy terminus MKK1 antibody was generated using the synthetic peptide GQDADGSTSPRSQEP. The amino-terminus MKK1 Ab was generated using a GST-fusion proteins containing 78 amino acids coded by the Smal to BG12 fragment of the MKK1 gene. The anti-carboxy terminus MKK2 Ab was made using a synthetic peptide corresponding to the sequence QQLSSIEPLREKDKH.

MKK1 and MKK2, cloned into the pBluskript plasmid, were transcribed and translated in the presence of ³⁵S-methione using standard methods.

Following protein synthesis MKK1 and MKK2 were immunoprecipitated (i.p.) with the appropriate rabbit antibodies (Ab) in the presence of SDS. Figure 5 shows immunoprecipitation of *in vitro* transcribed and translated MKK1 and MKK2 proteins.

9. Example: Expression of MKK1 Anti-sense Sequences

Bone Marrow elements isolated from mice treated with 5-flurourocil 6 days prior to harvest were infected with retroviruses containing constructs expressing MKK1, antisense MKK1 (a truncated 5' EcoRI-PvuII fragment cloned in the reverse orientation) or the empty retroviral vector (mock). Following infection, cells were cultured and analyzed for the level of acetylcholinesterase (AChE) as previously described, measured as optical density at 414 nm (Hill, Exp. Hematology 20:354-360 (1992)). A higher optical density reading indicates a greater AChE level and correlates with increased megakaryocyte growth and differentiation. Levels of the murine MKK1 protein were determined by metabolically labeling cells with ³⁵S-methionine for 12 hours at the end of the experimental period. Following labeling, cells were lysed and MKK1 protein was isolated by two cycles of immunoprecipitation using anti-amino terminus MKK1 antibody. The proteins were resolved by polyacrylamide gel electrophoresis and visualized by autoradiography.

The retroviral construct used (pSR/MSV-Tkneo) was previously described (Mol. Cell. Biol. 11:1785-1792 (1991)). The MKK1 sense construct represents the full length gene lacking the poly-adenylation sequences. The MKK1 antisense construct represents the 5' fragment EcoRI-PvuII cloned in the reverse orientation. Both the sense and antisense constructs

are driven by the retroviral long terminal repeat (LTR).

The results of the experiment, as shown in Figures 6A-6B, indicate that expression of the MKK1 anti-sense sequences in the cultured bone marrow elements is associated with decreased expression of MKK1 and decreased levels of AChE, an indicator of megakaryocyte growth and differentiation.

10. Example: MKK1 Protein Tyrosine Kinase Activity

The protein tyrosine kinase activity of MKK1 was demonstrated through the incorporation of ^{32}P in poly (Glu-Tyr) substrate by MKK1. The MKK1 used to demonstrate protein tyrosine kinase activity was obtained from 293 cells transiently overexpressing MKK1.

10.1. Materials and Methods

293 cells were transiently transfected with pCMP1-MKK1 (White, et al. J. Biol. Chem. (1987) 263:2969-2980), an expression vector containing nucleic acid encoding MKK1 operably linked to the CMV promoter. The 293 cells were harvested 48 hours after transfection and lysed in 1 ml of lysis buffer (20 mM tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.1 mM sodium orthovanadate, 0.1 mM PMSF) per one 10 cm culture dish. The lysates were collected by centrifugation at 20,000 g for 30 minutes. Control lysates were prepared in the same way as non-transfected 293 cells.

One ml aliquotes of the MKK1 transfected cell lysates and the control lysates, respectively, were incubated under constant agitation for 2 hours at 4°C with 5 μl of polyclonal antiserum raised against a GST fusion protein containing amino acid residues 25-223 of the N-terminus of MKK1 and 15 μl of Protein A-

Sepharose. The resultant MKK1 immunoprecipitates were spun down and washed with the lysis buffer twice; 50 mM tris-HCl [pH 7.4], twice; and either Mg^{2+} - or Mn^{2+} -containing kinase buffers (50 mM tris-HCl [pH 7.4], 5 mM of $MgCl_2$ or 5 mM $MnCl_2$, respectively), once. After the washes, the MKK1 immunoprecipitates were resuspended in 40 μ l of either the Mg^{2+} - or Mn^{2+} -containing kinase buffers containing 1 mg/ml of poly (Glu-Tyr) 4:1 (Sigma, P-0275) and 10 μ Ci of gamma-32P-ATP (Amersham) and incubated for 20 minutes at 37°C. 10 μ l aliquotes of the kinase reactions were spotted on glass paper filter (1205 Betaplate cassette filtermat, Beckman), and the filters were washed for 1.5 hours in three, sequential 100 ml volumes of (10% trichloroacetic acid, 10 mM sodium pyrophosphate). The washed filters were dried and analyzed by scintillation counting using BS Betaplate liquid scintillation counter (Beckman).

10.2. Results

Immunoprecipitates from MKK1 transiently transfected cells exhibited a 20-50-fold increase in counts over control cells, while no significant increase in counts was detected for immunoprecipitates from non-transfected cells or for MKK1 kinase assays carried out in the absence of poly (Glu-Tyr). Higher tyrosine kinase activity of MKK1 was observed in the presence of manganese than in the presence of magnesium.

11. Example: Biological Activity of MKK1

In order to assess the biological activity of MKK1, a murine megakaryocytic cell line (L-8057) was infected with a retrovirus containing nucleic acid encoding MKK1 and the selectable drug-resistance marker, neomycin. Cell growth of the MKK1 expressing

L-8057 cell lines and the ability of MKK1 expressing L-8057 cell lines to induce differentiation in the presence of tetradecanoyl phorbol acetate (TPA) was determined. The MKK1 expressing L-8057 cell lines
5 were incubated with various cytokines to determine if any cytokine had the ability to block growth inhibition by MKK1.

11.1. Materials and Methods

10 11.1.1. Retroviral Infection of L-8057 cells

Aliquotes of about 2×10^6 L-8057 cells (L-8057 cells were obtained from Dr. Yoji Ishida at the Iwate Medical University, Morioka, Japan) (about 1.5 ml of 8
15 $\times 10^5$ cells per ml in 15 ml conical tube) were prepared and collected by centrifugation. The collected cells were resuspended in 1.5 ml of viral stock of either ψ^- Eco msvTKneo MKK1 full length, designated V25, or ψ^- Eco msvTKneo, designated V6, (pPSR-a described in
20 Muller et al. (1992) Mol. Cell. Bio. 11:1785-1792) containing 1.5 μ l per ml of 6 mg/ml polybrene (Sigma, H 9268) and incubated at 37°C/5% CO₂ for 3 hours, with swirling every 30 minutes. The cells were then collected by centrifugation. An additional 1.5 ml of
25 viral stock containing polybrene was added and the cells were incubated for an additional 3 hours as described above. The cells were collected by centrifugation, resuspended in 2 ml of standard L-8057 medium (20% fetal bovine serum (FBS), 40% RPMI, 40%
30 IMDM (Gibco)), placed in 6 well plates and incubated at 37°C for 2 days.

The cells were collected by centrifugation, resuspended in 1 ml of medium containing 1 mg/ml G-418 and placed in 6 cm dishes with 5 ml of medium
35 containing G-418.

Non-infected L-8057 cells grown in media containing G-418 (1×10^5 cells per ml in 10 cm dish) were used as a control.

Following drug-selection in G-418 and cell expansion, cells counts were made on days 5 and 6. Expression of MKK1 was verified by Western Blot.

11.1.2. Cell Growth Measurement of MKK1 Expressing L-8057 Cells

Cell growth of the MKK1 expressing L-8057 cell lines, designated V25A and V25B, along with mock-infected L-8057 cells and control cells was measured. Duplicate 6 well dishes plated at a cell density of 1×10^5 /ml or 3.3×10^4 /ml cells were prepared in 1% Nutridoma (Boehringer Mannheim). Cell counts were taken at days 5 and 6.

<u>Day 5 cell counts:</u>		<u>Day 6 cell counts:</u>		<u>vol. 2×10^5</u> <u>cells</u>
CNTL	1.9×10^6 /mL	CNTL	1.6×10^6 /mL	0.125 ml
V6	5.2×10^5 /mL	V6	4.0×10^5 /mL	0.5 ml
V25A	0.8×10^5 /mL	V25A	0.8×10^5 /mL	2.5 ml
V25B	1.6×10^5 /mL	V25B	1.0×10^5 /mL	2.0 ml

11.1.3. Growth Factor Response of MKK1 Expressing L-8057 Cells

The cells from one dish each of L-8057 cells infected with a retrovirus containing full length MKK1, designated V25A, and empty vector, designated V₆₋₁ were collected through centrifugation, washed with 10 ml of (IMDM, 1% Nutridoma, glutamine (Gibco), penicillin-streptomycin (Gibco) recentrifuged and resuspended in 2 ml of same medium. The cells were diluted to 2×10^5 cells / ml and 50 μ l (or 10^4 cells) were added per well of a 96 well plate and incubated

for 24 hours under conditions of serum starvation. After serum starvation, rat stem cell factor, cKit ligand, IL-3, IL-6, IL-11, IL-1 β , EPO, human MPL ligand, Spleen Cell Conditioned Medium (IL-3, IL-6) to
5 a volume of 10%, WEHI 38 Conditioned Medium (IL-3, GM-CSF) to a volume of 10%, and fetal bovine serum to a volume of 10% (as a positive control) were added to the cells. The cells were allowed to grow for three
10 days and cell growth was measured in a standard MTT (tetrazolium) assay (Mosmann, J. Imm. Meth. (1983) 65:55-63)

11.2. Results

11.2.1. Cell Growth

15 The highest MKK1 expression was observed in cells designated L-8057 V25A. No expression was observed in cells infected with the empty vector control, designated L-8057 V6. The growth rate was then
20 examined under growth limiting conditions, i.e., in serum-free media. Compared with the empty vector control, cells infected with MKK1 grew at a much slower rate, see Figure 12.

11.2.2. Cytokine Stimulation

25 Stimulation of the MKK1 infected L-8057 cells with a panel of cytokines, including rat stem cell factor (SCF), IL-3, IL-6, IL-11, IL-1 β and EPO, revealed that only rat stem cell factor (SCF) was
30 capable of overcoming the inhibition of proliferation produced by the hyperexpression of MKK1, see Figure 13. The control cells also responded to SCF. The percent increase in the growth of the control cells treated with rat SCF and the MKK1 infected cells treated with rat SCF was similar. The data suggest
35 that rat stem cell factor does not have the same signalling transduction pathway as MKK1.

11.2.3. Cell Differentiation

In order to test the effect of MKK1 on megakaryocyte differentiation, a murine megakaryocytic cell line (L-8057) was engineered to express MKK1, as described in Section 11.1.1. Induction of polyploidy (cells with DNA content greater than 4N) is a hallmark of megakaryocytic differentiation. L-8057 can be induced to differentiate (measured by induction of polyploidy and expression of the enzyme acetylcholinesterase in murine systems) by treatment with tetradecanoyl phorbol acetate (TPA) (Ishida et al., Exp. Hematol. 21:289-298, 1993). Figure 14 demonstrates the effect of TPA on either control cells or cells that express MKK1. Both control cells and MKK1 expressing cells became polyploid to the same extent in response to treatment with TPA for 3 days. This data suggest that induction of differentiation is not affected by the presence of MKK1.

Various modifications of the invention, in addition to those shown and described herein, will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims. It is also to be understood that all base pair sizes given for nucleotides are approximate and are used for purposes of description.

All references cited herein are hereby incorporated by reference in their entirety.

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